SELECTIVE LIPID HYDROLYSIS BY *GEOTRICHUM CANDIDUM* NRRL Y-553 LIPASE

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SUMMARY

A crude preparation of lipases from the fungus *Geotrichum candidum* NRRL Y-553, which shows specificity toward **cis**-9 unsaturated fatty acids, was used to hydrolyze natural fats and oils, with the goal of producing industrially useful fatty acid and/or mono- and diacylglycerols products. Tallow and soy oil were hydrolyzed, producing free fatty acids high in oleic or **cis**-9 unsaturates, respectively. Hydrolysis of rapeseed oil produced acylglycerols enriched in erucic acid.

INTRODUCTION

The lipases produced by the fungus *Geotrichum candidum* specifically hydrolyze triacylglycerols containing unsaturated fatty acids with a **cis**-double bond in the nine position (oleic (18:1)[1]; linoleic (18:2); linolenic (18:3); palmitoleic (16:1)) in preference to those containing the saturated fatty acid, stearic acid (18:0) (Jensen, 1974). Our goal is to use *G. candidum* lipase selectively to produce unsaturated fatty acids from agricultural fats and oils. However, since these natural triacylglycerols contain significant amounts of palmitic acid (16:0) in addition to 18:0, a lipase is required which selectively releases the **cis**-9 unsaturates in preference to both 16:0 and 18:0. We have previously characterized various strains of *G. candidum* lipase (Baillargeon *et al.* 1989), and found that the lipases of *G. candidum* NRRL Y-553 had such a specificity. We found that crude NRRL Y-553 lipases hydrolyze 4-methylumbelliferyl esters of oleic acid 20 times faster than those of palmitic acid, and that purification and partial separation of the multiple forms of lipase present did not improve the specificity (Baillargeon and McCarthy, 1991). We describe here the extension of our work with this lipase to the hydrolysis of natural fats and oils.

MATERIALS AND METHODS

Materials. Triacylglycerols used were: high erucic acid rapeseed oil (Agro, Des Plaines, IL)[2], bleached tallow (Chemol Inc., Greensboro, NC), and soy oil (food grade). All other materials were reagent grade or better.

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[1]Abbreviations: Free fatty acids, FFA. Fatty acid abbreviations indicate number of carbon atoms:number of double bonds: eicosenoic, 20:1; erucic, 22:1; linoleic, 18:2; linolenic, 18:3; oleic, 18:1; palmitic, 16:0; palmitoleic, 16:1; stearic, 18:0.

[2]Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Lipase production. *G. candidum* NRRL Y-553 was obtained from the Agricultural Research Service Culture Collection (Peoria, IL). The culture was maintained as previously described (Baillargeon and McCarthy, 1991), and grown in a 2.5 L BioFlo III fermentor (New Brunswick Scientific Co., Edison, NJ) using a medium (pH 7.0) of 5.0% bacto-peptone (Difco Laboratories, Detroit, MI), 0.1% each of $MgSO_4 \cdot 7H_2O$ and $NaNO_3$, and 0.75% soy oil (Sigma Chemical Co., St. Louis, MO). The fermentation was run without pH control at 30°C, 400 rpm and 1.5 L/min aeration. Lipase activity was monitored with time, and when the activity was maximized (about 30 U/ml), 400 ppm Biocryl BPA-1000 (Supelco, Bellefonte, PA) was added to aid centrifugation, and the medium was centrifuged for 200,000 g x min. The supernatant was frozen or lyophilized for later use.

Enzyme assays. Lipase activity and specificity, and protein content were assayed as previously described (Baillargeon, 1990). A unit of lipase activity is defined as the release of one micromole of free fatty acid (FFA)/min as measured by pH stat titration (pH 8.2) of a 10% olive oil-gum arabic emulsion at room temperature.

The variation of lipase activity with pH was measured by varying the pH of the emulsion and pH stat titration. The activity under standard conditions (pH 8.2) varied between 20-30 U/ml with different emulsions. This activity was defined as 100%, and the data were normalized to allow comparisons among different emulsions. The lipase activity was corrected for blank activity where this was significant (pH 9.2 and above). The rate of hydrolysis was measured after a lag time whose length depended upon pH; at pH 6.6, the rate significantly increased after 60 minutes. The lag time decreased with increasing pH; at the most basic pH values, there was no lag. The measurements in the basic pH range were stopped due to the increasing blank activity and electrode limitations.

The stability of lipase as a function of temperature was measured by incubation of a 100 mg/mL sample of lyophilized enzyme in water (pH 6.7) at 45°C. Aliquots were removed and assayed under standard conditions.

Gas liquid chromatography. Samples were transesterified with NaOMe/MeOH, and the methyl esters were analyzed against gravimetric standards (Supelco Inc., Bellefonte, PA) using a Chrompack gas chromatograph with an SP2330 capillary column (0.25 mm i.d. x 30 m) operated at 190°C and a Hewlett Packard 3390A integrating recorder (Avondale, PA).

Enzymatic hydrolysis. The lipase-catalyzed hydrolyses were conducted as follows: $0.3000 \pm .0001$ g of fat/oil (molecular weight of fat/oil estimated from the methyl esters analysis) was combined with 5.0 ml of 0.1 M Tris (pH 8.0), 0.25 g of gum arabic, and 100 mg of lyophilized Y-553. The mixture was sonicated for 30 sec in a Ramco ultrasonic bath, stirred at 35°C for various periods of time, and then separated as previously reported (Sonnet and Gazzillo, 1991) into two fractions containing methyl esters (representing product acids) and recovered, unhydrolyzed acylglycerols. These were then analyzed by gas liquid chromatography as described above.

RESULTS

Characterization of lipase preparation. The centrifuged fermentation supernatant had 27 U/mL lipase activity, 41 μg protein/mL, and a specificity 18:1/16:0 of 18 ± 4. The lyophilized supernatant had a lipase activity of 0.49 U/mg powder, contained 0.25% protein, and exhibited a specificity 18:1/16:0 of 19.

The lipases were active on olive oil emulsion over a wide basic pH range (Figure 1), with a maximum at pH 8.2, and a sharp decline in activity around pH 6.6.

The stability of the lipases at 45°C is shown in Figure 2. Although activity decreased rapidly, a stable species representing about 10% of the original activity remained upon longer incubations. All activity could be eliminated by boiling the lipase. When the data were fit to the sum of two first order reactions by nonlinear regression, rate constants of $0.0885 \pm .0114$ h^{-1} ($t_{1/2} = 8$ h) and $0.0049 \pm .0034$ h^{-1} ($t_{1/2} > 100$ h) were obtained.

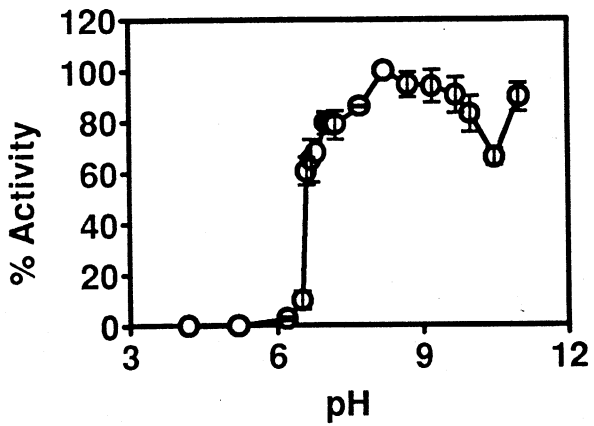

Figure 1. Effect of pH on the activity of *G. candidum* NRRL Y-553 lipases. The initial rate of hydrolysis of an olive oil-gum arabic emulsion at each pH was measured and normalized to the rate of pH 8.2.

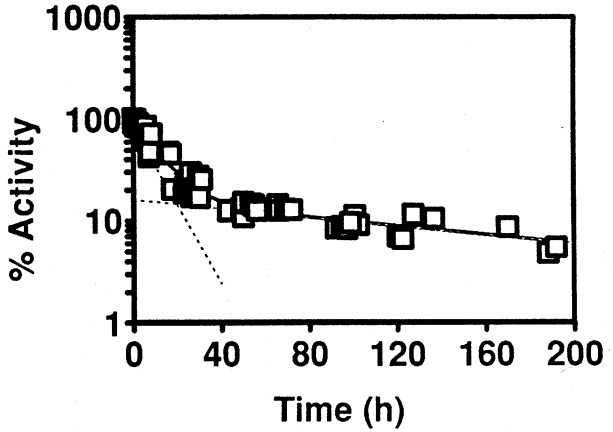

Figure 2. Activity of *G. candidum* NRRL Y-553 lipases on an olive oil-gum arabic emulsion as a function of lipase incubation time at 45°C. The data are fit to the sum of two first order rate expressions by nonlinear regression (solid line). The two calculated rates are shown by dashed lines.

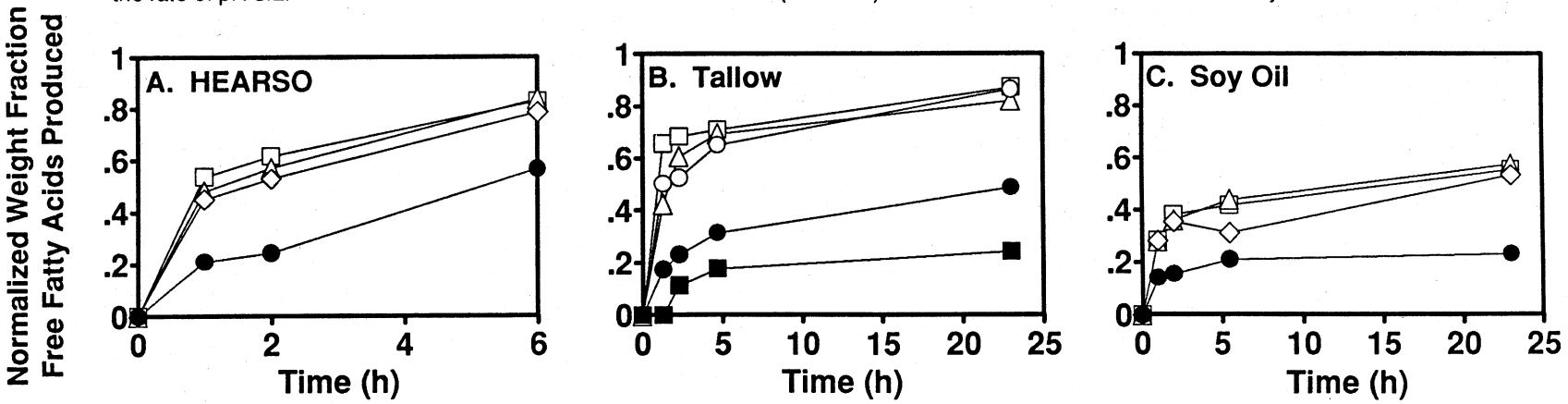

Figure 3. Hydrolysis of HEARSO (A), tallow (B), and soy oil (C) by *G. candidum* NRRL Y-553 lipases. The product free fatty acids are normalized relative to their original composition: 16:1, ○; 18:1, □; 18:2, △; 18:3,◇; 16:0, ●: 18:0, ■.

Hydrolysis of high erucic acid rapeseed oil (HEARSO). The hydrolysis of HEARSO by *G. candidum* NRRL Y-553 lipase is shown in Figure 3A. The FFA produced were normalized to their composition in the unreacted oil, and plotted as a function of time. The **cis**-9 fatty acids were rapidly released; 82% reacted in 6 hours. Palmitic acid was released more slowly, while hydrolysis of eicosenoic acid (20:1) was not detected. Only a trace of erucic acid (22:1) was released (below reliable detection limits).

Hydrolysis of tallow. The hydrolysis of tallow over 23 h by *G. candidum* NRRL Y-553 is shown in Figure 3B. The **cis-9** unsaturated fatty acids were released most rapidly, followed by 16:0, and 18:0 in lesser amounts. The fatty acids 14:0 and **trans**-9 18:1, composing 3% each of the tallow, were released only in trace amounts (data not shown).

Hydrolysis of soy oil. The hydrolysis of soy oil over 23 h by *G. candidum* NRRL Y-553 is shown in Figure 3C. The **cis**-9 unsaturated fatty acids were released more rapidly than 16:0 acid. Only trace amounts of 18:0 were released (data not shown). While the initial rate of hydrolysis of soy oil was similar to that of tallow, the rate rapidly decreased; at 23 h, 55% of the **cis**-9 fatty acids in soy oil had been hydrolyzed, compared to 85% in tallow. This may be due to the presence of endogenous phospholipid (Wang and Gordon, 1991) or other inhibitors in the food-grade oil.

DISCUSSION

G. candidum NRRL Y-553 lipases could be used to produce oleic acid from tallow or mixed **cis**-9 unsaturated fatty acids from soy oil. In tallow hydrolysis, at 36.2% conversion, the product fatty acids were 86.4% unsaturated. At 61.2% conversion, the product had declined to 71.4% unsaturation. This decline in product purity is the inevitable result of kinetic resolution; to obtain a pure product, a very high relative specificity or control of the degree of conversion is required. Nevertheless, tallow, which contains little polyunsaturation, may offer a source of relatively pure oleic acid using hydrolysis by NRRL Y-553 lipases carried to low conversion. Soy oil that originally contained 83.1% unsaturated fatty acids was converted after 50% hydrolysis to fatty acids that were 94.4% unsaturated.

G. candidum NRRL Y-553 lipases also could be used to enrich mono- and diacylglycerol products in fatty acids which the lipase discriminates against, such as 22:1. In the hydrolysis of HEARSO, the recovered (unreacted) acylglycerols at 43% conversion were enriched to 66.2% of 22:1, compared to 37.8% initially. Other examples of enrichment of acylglycerols by selective removal of fatty acids have been reported (Ergan, 1991; Hills *et al.*, 1989).

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